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Location, Exposure, and Conservation of Neutralizing and Nonneutralizing Epitopes on Human Immunodeficiency Virus Type 2 SU Glycoprotein

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Eleven rat monoclonal antibodies (MAbs) that recognize the SU glycoprotein of human immunodeficiency virus type 2 (HIV-2) ROD were produced and characterized. Binding sites for eight of these MAbs were mapped to epitopes within the C1, V1/V2, C2, and V3 envelope regions. The three other MAbs defined at least two conformation-dependent, strain-specific epitopes outside V1/V2, V3, and the CD4-binding site. The MAbs were used to probe the tertiary structure of oligomeric envelope glycoprotein expressed on the surfaces of infected cells. Epitopes at the apices of V2 and V3 were exposed on the native molecule, whereas other epitopes on V1/V2, C1, and C2 were hidden. The MAbs defined three neutralization targets on exposed domains: two linear epitopes in the V2 and the V3 loops and one conformational epitope outside V1, V2, and V3.

Human immunodeficiency virus type 2 (HIV-2) (13, 25) causes AIDS throughout West Africa in Guinea Bissau and Cape Verde Islands (14), as well as The Gambia (63), Senegal (36), The Ivory Coast (18), and Mali (59). Evidence that HIV-2 had spread to India first emerged in 1990 (61), with infection in Southern and Southwestern India occurring in parallel with the spread of HIV-1 in both the heterosexual and the homosexual populations (2, 24, 40, 55, 58, 62). This present outbreak, together with the presence of HIV-2 in Europe, particularly in Portugal and France (66), predicts further spread of the virus.

Envelope glycoproteins on the virus particle initiate the first events in the virus life cycle, binding to the cell surface receptors on target cells and mediating subsequent events that lead to fusion of the viral and cellular lipid membranes. At present, resolution of the crystal structure of the envelope glycoproteins of HIV or simian immunodeficiency virus (SIV) is not in sight, and structural analyses of these glycoproteins rely heavily on probing with monoclonal antibodies (MAbs). This approach has enabled a three-dimensional model of the HIV-1 envelope glycoprotein, gp120, to be developed (50).

Serum from HIV-2-infected humans often cross-neutralize SIV (60) and HIV-1 (75). Characterization of this neutralization may lead to a cross-protective vaccine effective against both HIV-1 and HIV-2. The antibody neutralization targets on HIV-1 and SIV envelopes are well-characterized and include regions in the outer envelope glycoprotein gp120 and the transmembrane glycoprotein gp41. For HIV-1, linear epitopes have been demonstrated in V2 (16, 22, 47, 76) and V3 (8, 9, 30, 31, 43, 77), as well as in gp41 (54). In addition, neutralizing MAbs directed to nonlinear, conformationally sensitive epitopes that block gp120 binding to the CD4 receptor have been generated (35, 41, 64, 67, 69, 71). Another conformationally sensitive epitope which becomes accessible to antibody only after the viral envelope binds to CD4 has been described. This region maps close to but outside the CD4-binding site, and it is not directed to the V3 loop (34, 70).

For SIV, linear neutralization epitopes have been described for the V2 (3, 38) and for the V4 domains (72) as well as for the transmembrane glycoprotein, gp41 (39). However, peptides representing the V3 loop of SIV have failed to induce neutralizing antibodies (33, 38). More recent studies have suggested that the V3 loop may be involved in a neutralizing epitope which is dependent on the envelope conformation (32).

Attempts to map neutralization epitopes in HIV-2 have had limited success. Although peptides derived from the SIV V2 domain induced antibodies that neutralized SIV (3, 38), equivalent peptides failed to induce neutralizing antibodies to HIV-2 (1, 56). It has been controversial as to whether the V3 domain in HIV-2 represents a neutralization target for HIV-2 (1, 4, 5, 42, 73). However, a recent report describes a weakly neutralizing MAb induced by a V3 loop peptide (44).

Here, we describe rat MAbs raised to recombinant baculovirus-derived envelope glycoprotein of HIV-2 ROD as well as to a V3 loop. MAbs that map to C1, V1, V2, C2, and V3 and to conformation-dependent epitopes are characterized, and we describe the locations and exposure, conservation, and neutralization properties of their epitopes.

MATERIALS AND METHODS

Viruses. HIV-2 LAV-2_{ROD} (HIV-2 ROD) was derived from a molecular clone, pROD23, which was kindly provided by Agnes Cordonnier. The CBL-22 strain was isolated in our laboratory (63). pSBL/ISY (ISY), a molecular clone of SBL6669, was kindly provided by Genoveffa Franchini (21). All other virus strains have been previously described (10, 11, 48).

Cells. Molt 4 clone 8, and C8166 cells have been described before (12). Sp.f.9 cells derived from *Spodoptera frugiperda* were used to propagate baculovirus constructs (20).

Preparation of hybridomas and screening of supernatants. CBH/Cbi rats were immunized three times at 21-day intervals, via Peyer's patches, with recombinant baculovirus-derived gp105 or V3 loop peptide circularized via the terminal cysteine residues (HIV-2 ROD). The immunogens were emulsified in complete Freund's adjuvant for the first inoculation and in incomplete Freund's for subsequent immunizations. Three days after the last immunization, mesenteric lymph node cells were fused with the rat myeloma line, Y3-Ag 1.2.3. (17). Supernatants were screened for antibodies to the immunizing antigen by enzyme-linked immunosorbent assaying (ELISA) and for binding to methanol-acetone (1:1)-fixed HIV-2 ROD-infected molt 4 cells as well as recombinant antigen gp105 by ELISA (see below). Mouse antibodies specific for rat immunoglobulin (Ig) heavy chains (γ1, γ2a, γ2b, γA, and γM) were used to determine the isotypes of the MAbs by radioimmunoassaying. Cloned MAbs were tested for binding to wells coated with each IgG-specific antibody. Bound MAbs were detected with I^{125}-labelled rabbit anti-rat IgG (mouse-absorbed) STAR 45 (Serotec).

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ELISA. Hybridoma supernatants were tested for binding to recombinant gp105, which was captured by sheep antibody specific for the C terminus of gp105 (D7335; Alto Bioreagents). After incubation for 1 h at room temperature, the plates were washed three times in wash buffer (phosphate-buffered saline [PBS], 0.1% bovine serum albumin [BSA], 0.05% Tween 20). Bound rat antibody was detected with goat anti-rat conjugated to horseradish peroxidase (Sera-Lab) by incubation at room temperature for 1 h. The plates were washed three times in wash buffer, and bound antibody was detected by the addition of TMB (Sigma), which yielded a soluble blue product over 20 min. Acidification with 0.5 M H_2SO_4 solution stopped the reaction and resulted in a yellow color when read at 450 nm.

Radioimmunoassay competition assays. I^{125}-MAbs at half-maximal binding concentrations were mixed with equal volumes of unlabelled MAb (10 μg/ml). A 50-μl aliquot of the mixture was then tested for binding to D7335-captured gp105 on radioimmunoassay flexiplates. The amounts of bound antibody were determined, and the percentage binding of labelled antibody was calculated (17).

Immunostaining with β-galactosidase conjugates. Molt 4 cells chronically infected with appropriate HIV-2 strains were washed twice in serum-free medium (RPMI 1640). A total of 3×10^5 cells per ml were added to 24-well trays (1 ml per well) that had been pretreated with poly-L-lysine (50 μg/ml [Sigma]) for 1 h and were washed thoroughly in serum-free medium. The cells were allowed to attach for 30 min at 37°C before being washed once in serum-free medium to remove unbound cells and being fixed in methanol-acetone. Fixed cells were washed again in PBS. Appropriate antibodies were added in PBS containing 1% fetal calf serum (PBS-FCS) and were incubated at room temperature for 1 h before being washed twice in PBS-FCS. For detection of rat antibodies, we then added an anti-rat IgG β-galactosidase conjugate (Euro-path Ltd.). After incubation for 1 h at room temperature, cell layers were washed in PBS and β-galactosidase substrate (48) was added. Positive cells stained blue.

Immunofluorescence. Assays were done on both live and fixed-cell preparations. Cells to be fixed (10^6 per sample) were washed twice in PBS, were resuspended in PBS plus 1% azide (5 mM), and were fixed for 2 min in cold methanol-acetone (or in 5% Formalin). Fixed cells were washed in PBS plus azide and pelleted. Unfixed live cells were washed once in PBS and resuspended at 10^6 cells per ml of PBS plus 1% azide. Cells were dispensed into tubes (1 ml per tube) and pelleted. Appropriate dilutions of antibody were added to cell pellets, and the cells were resuspended and allowed to stand at room temperature for 1 h. The cells were washed twice, and an appropriate fluorescein isothiocyanate (FITC) conjugate added. An anti-rat IgG FITC conjugate (Genosys) was added for detection of rat antibodies while mouse antibodies were specifically detected by an anti-mouse IgG FITC conjugate that did not recognize rat antibodies (Serotec). After a further incubation, the cells were washed again and finally resuspended in 500 μl of 5% formalin in PBS. Immunofluorescence intensity was detected and measured with a fluorescence-activated cell sorter (FACScan; Becton Dickinson).

Western blotting (immunoblotting). Preparations of cell-free virus were used as antigen for Western blot analysis. C8166 cells were infected with viral supernatant and incubated for 1 week (37°C) before being mixed with fresh C8166 cells (5 ml of infected cells plus 15 ml of uninfected cells at 10^5 cells per ml). After a further incubation for 3 days, the viral supernatant was pelleted at 20,000 rpm in a rotor (6 by 14 ml) in a Sorvall OTD 55B ultracentrifuge. The liquid layer was discarded, and virus was resuspended in PBS and stored at −70°C. Virus samples were analyzed by standard Western blotting techniques. Briefly, proteins were separated on 0.1% SDS–10% polyacrylamide gels and were electrophoretically transferred to nitrocellulose filters. The filters were washed in Tris-buffered saline (TBS) alone and then in TBS containing 0.1% Tween 20 (TBS-Tween 20). The blots were then immersed in TBS-Tween 20 with 5% nonfat milk (Marvel) for 1 h and then with antibody (5 μg/ml) in fresh TBS-Tween 20–milk–20% fetal lamb serum for 2 h at room temperature. The blots were washed in TBS-Tween 20 three times before addition of goat anti rat Ig-horseradish peroxidase conjugate (Serotec) at a 1/500 dilution in TBS-Tween 20–milk–lamb serum. After 2 h of incubation at room temperature, the blots were washed three times in TBS-Tween 20 and a further three times in TBS alone. The blots were developed with Amersham ECL reagent.

Epitope mapping with chimeric HIV-1 and HIV-2 envelope constructs. Chimeric gp105 envelope glycoproteins (derived from HIV-1 LAI and HIV-2 ROD) have been described previously (53). Baculoviruses containing each construct were added to Sp.f.9 cells in 24-well trays. After 2 days of incubation, infected cells were washed with PBS and fixed in unlabelled methanol-acetone (1:1) for 10 min. After further washes in PBS and PBS-FCS, the infected cells were immunostained with appropriate MAbs and then with anti-rat β-galactosidase-conjugated antibody as described above.

Epitope mapping with overlapping peptides. Three sets of overlapping peptides corresponding to the envelope sequence of $LAV\text{-}2_{ROD}$ were made. The peptides were prepared on a cellulose filter paper by using the SPOTS kit supplied by Genosys Biotechnologies, Inc. All peptides were 12 amino acids in length. The first set (set 1) covered the entire gp105 sequence, with each peptide overlapping the preceding one by six amino acids. Set 2 covered V1 and V2 from amino acids 86 to 186 (CVAMKCSSTE SSTGNNTTSK STSTTTTTPT DQEQEISEDT PCARADNCSG LGEEETINCQ FNMTGLERDK KKQYNETWYS KDVVC ETNNS TNQTQCYMNH C). Set 3 covered the V3 loop, from cysteine to cysteine (CKRPGNKIVK QIMLMSGHVF HSHYQPINKR PRQAWC).

With these last two sets, each peptide commenced 1 amino acid downstream from the previous peptide, thus overlapping with 11 of its 12 amino acids.

MAbs 28.8e, 44.5j, and 44.6i (groups D, C, and A, respectively [see Fig. 1]) were tested on the first set of peptides. MAbs 28.8c, 44.5k, 44.2g, and 44.1b (group B) had been shown to bind to a region including V1 and V2. These MAbs were tested for binding to peptide set 2.

MAbs 32.2f and 32.7g were both raised to a V3 loop peptide. These MAbs were finely mapped with peptide set 3.

Neutralization assays. A 40-μl volume of virus supernatant containing 100 50% tissue culture infective doses of virus was mixed with serial dilutions of purified antibody in a microtiter plate. The mixture was incubated at 37°C for 1 h, 100 μl of C8166 cells at 2.5×10^5 cells per ml was added, and this mixture was further incubated for 3 days. Neutralization titers were determined as the concentration of MAb (in micrograms per milliliter) required to reduce the number of syncytia induced by the input virus by at least 90%.

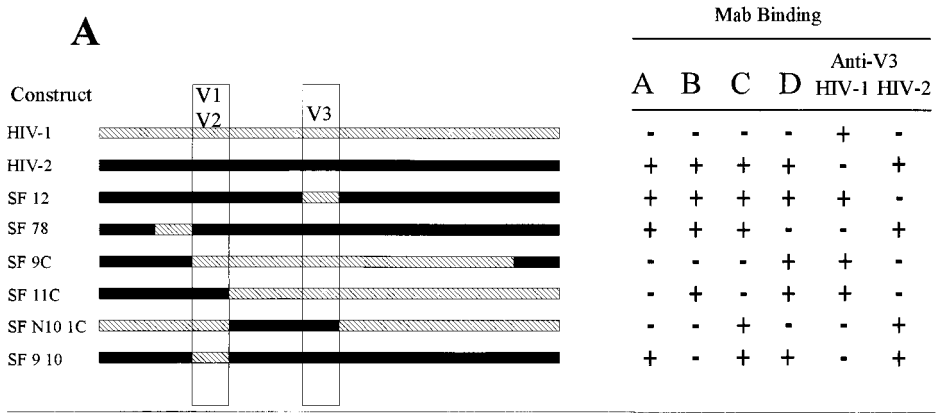
RESULTS

Production of MAbs to HIV-2 ROD envelope glycoprotein. We immunized three rats (strain CBH/Cbi) with recombinant baculovirus-derived HIV-2 ROD envelope protein, gp105 (rat nos. 25, 28, and 44), and a fourth rat (rat no. 32) with a peptide representative of the entire V3 loop region. The immunization protocols are outlined in Materials and Methods. Supernatants from the hybridoma fusions were tested for binding to fixed HIV-2 ROD-infected cells by using an anti-rat IgG β-galactosidase conjugate for detection (β-galactosidase assay). Additionally, an antigen-capture ELISA with recombinant gp105 was used for confirmation (Materials and Methods). Eleven independent MAbs that were positive in both assays were produced, and their isotype specificities were determined. Five MAbs have isotype specificity γ2a (25.8c, 28.8e, 44.5j, 44.2g, and 44.6i), four have specificity γ2b (25.3f, 28.3e, 44.1b, and 32.7g), and two have specificity γ1 (44.5k and 32.2f).

Mapping MAbs with envelope constructs chimeric between HIV-1 and HIV-2. MAbs were initially mapped by using baculovirus-derived constructs containing surface (SU) envelope glycoprotein that were chimeric between HIV-2 ROD and HIV-1 LAI (53). MAbs were tested for binding to insect cells infected with appropriate baculovirus constructs and fixed in methanol-acetone. Binding was determined by the β-galactosidase assay (Materials and Methods). The results are illustrated in Fig. 1. All MAbs bound to cells expressing the HIV-2 envelope and not to those expressing the HIV-1 envelope. One of the MAbs specific for the HIV-2 V3 loop (32.7g) was used to verify constructs that contained ROD V3 sequences (SF 78, SF N10/1C, and SF 9,10). An anti-HIV-1 MAb specific for the LAI V3 loop (46) bound specifically to cells expressing the HIV-1 envelope protein and only constructs containing HIV-1 V3 (SF 12, SF 9C, and SF 11C).

Figure 1 shows that apart from 32.7g, a MAb specifically raised to the HIV-2 ROD V3 loop, all of the MAbs bound to construct SF 12, in which the HIV-2 V3 loop is replaced with that of HIV-1 LAI. Thus, none of the MAbs raised to the whole envelope are specific to the V3 loop. On the basis of their binding profiles to the remaining chimeric SU envelopes, the MAbs were placed into four groups (A to D [Fig. 1B]). In construct SF 78, a small region immediately upstream of V1/V2 (amino acids 38 to 82) is substituted with that of HIV-1; however, this construct was still recognized by groups A (28.8e, 28.3e, and 25.3f), B (25.8c, 44.5k, 44.2g, and 44.1b), and C (44.6i), thus excluding this region from involvement in their epitopes. However, group D (44.5j) lost binding to this construct, suggesting that its epitope is either within this stretch of amino acids or was disrupted by its removal. Nevertheless, group D bound to SF 9C and to SF 11C, in both of which the carboxy terminus from amino acid 83 is replaced with the equivalent region of HIV-1 LAI; thus, the epitope recognized by group D lies within the region encompassed by amino acids 1 to 82.

Group A bound to construct SF 9,10 and SF 78, excluding V1/V2 and amino acids 32 to 82 from its epitope. Group A



B

Preliminary assignment of epitopes

Group	Mab	Location
A	28.8e, 28.3e, 25.3f	Not V1/V2,V3 nor 38-82 in C1
B	25.8c, 44.5k, 44.2g, 44.1b	V1/V2
C	44.6i	189-289, C2
D	44.5j	1-82, C1
Anti-V3	32.7g	V3

FIG. 1. Mapping MAbs with envelope constructs between HIV-1 and HIV-2. Chimeric LAI/ROD SU glycoprotein constructs in baculovirus were expressed after infection of sp.f.9 insect cells. MAb binding was tested after fixing cells in methanol-acetone (1:1). Binding was detected with an anti-rat IgG β-galactosidase conjugate as described in Materials and Methods, and positive binding (+) was indicated by blue staining. (A) MAb reactivity with envelope constructs; (B) MAbs grouped according to the preliminary locations of their binding sites.

could not be further mapped more closely than this by using these constructs; however, it can be concluded that group A antibodies (28.8e, 28.3e, and 25.3f) are not directed to V1/V2 or V3 or to amino acids 38 to 82 in C1.

Group B mapped to the V1/V2 domain, although the N-terminal 32 amino acids could not be excluded. They bind to constructs SF 12, SF 78, and SF 11C, all of which include the V1/V2 domain. In contrast, binding is lost for constructs (SF 9C, SF N10/1C, and SF 9,10) in which the V1/V2 domain is substituted for that of HIV-1 LAI.

The smallest HIV-2 fragment to which group C bound was SF N10/IC, which contains the C2 region from amino acids 189 to 289 (with the V3 loop already eliminated from involvement [see above]).

In summary (Fig. 1B), group A (28.8e, 28.3e, and 25.3f) maps to regions outside V1/V2, V3, and amino acids 38 to 82 in C1. Group B (25.8c, 44.5k, 44.2g, and 44.1b) maps within V1/V2. Group C (44.6i) maps to C2 within amino acids 189 to 289, and group D (44.5j) maps to within amino acids 1 to 82.

Fine mapping of MAbs with overlapping peptides. We prepared three sets of overlapping 12-mer peptides to map the MAbs more finely. The first set covered the entire envelope of HIV-2 ROD and was used to map groups A, C, and D. Each peptide overlapped the previous one by six amino acids. The second peptide set was confined to the V1/V2 region to focus on the epitopes recognized by group B. These peptides each began one amino acid further on, compared with the previous peptide, to provide maximum information on the core epitope required for recognition.

Attempts to map MAb 28.3e or 28.8e in group A with the overlapping peptides failed. Thus, it is likely that these MAbs do not recognize a linear epitope but recognize a conformational epitope on the envelope. This proposal is further supported by the Western blot result that shows that these MAbs failed to recognize severely reduced or denatured protein (data not shown).

All of the other MAbs bound at least one 12-mer peptide and thus recognized linear epitopes. These results confirmed and extended the results shown in Fig. 1. Epitopes are shown in Table 1 and are illustrated in the context of a cysteine loop map of gp105 (19, 29) in Fig. 2. MAb 44.5j recognized amino acids 43 to 54 in the C1 domain. MAb 28.8c binds to amino

TABLE 1. Epitope recognition of MAbs to HIV-2 envelope glycoprotein gp105[a]

MAb	Amino acids	Epitope	Envelope domain
44.5j	43–54	DDYQEITLNVTE	C1
25.8c	125–133	SEDTPCARA	V1
44.5k	140–148	GEETINCQ	V2
44.2g	149–154	FNMTGL	V2
44.1b	167–175	YSKDVVCET	V2
44.6i	223–234	DTNYSGFAPNCS	C2
28.3e		Conformation dependent	Not V1, V2, or V3
28.8e		Conformation dependent	Not V1, V2, or V3
25.3f		Conformation dependent	Not V1, V2, or V3
32.2f	304–311	LMSGHVFHSHYQ	V3
32.7g	306–311	SGHVFHSHYQ	

[a] MAbs were first mapped by using chimeric envelopes between HIV-2 ROD and HIV-1 LAI to locate broad regions. Finer mapping was done with overlapping peptides derived from specific regions. MAbs 28.3e, 28.8e, and 25.3f did not bind to any of 80 overlapping peptides encompassing the entire HIV-2 ROD envelope and thus could not be mapped further.

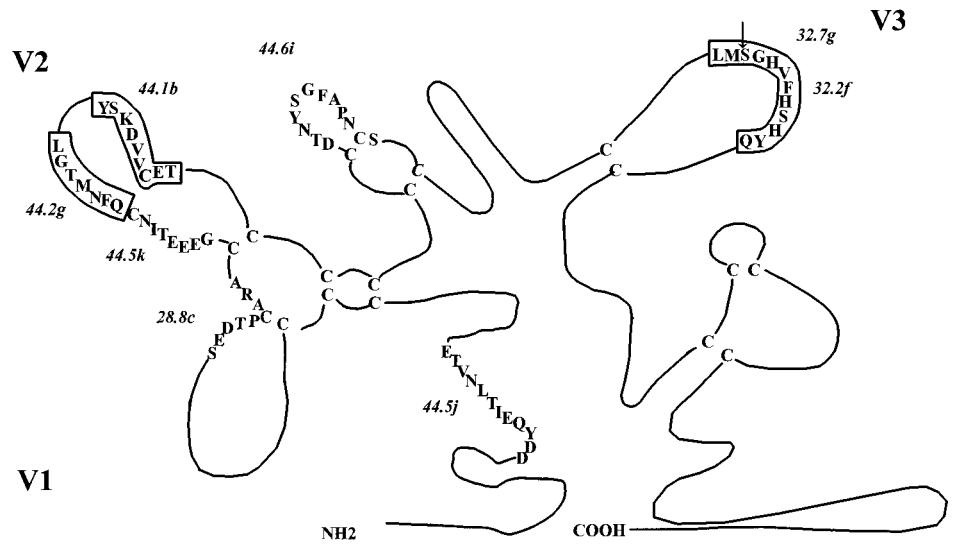


FIG. 2. Locations of linear epitopes of rat MAbs on a cysteine loop map of HIV-2 SU glycoprotein (19, 29). The arrow delimits the 32.7g epitope.

acids 125 to 133 in the V1 region. MAb 44.5k binds to amino acids 140 to 148, 44.2g binds to amino acids 149 to 154, and 44.1b binds to amino acids 167 to 175, all within the V2 domain. Finally, MAb 44.6i maps to a short loop in the C3 region (amino acids 223 to 234).

Fine mapping of V3 loop-specific antibodies. A third set of peptides was designed to map the minimum epitope recognized by the two V3-specific MAbs. Each 12-mer overlapped the previous peptide by 11 amino acids. As expected, MAbs 32.7g and 32.2f mapped to linear domains within the V3 loop. However, the core epitope recognized by MAb 32.7g was smaller than (and contained within) that for 32.2f (Table 1), indicating that the MAbs recognize distinct but overlapping epitopes.

One of the V3 loop MAbs recognized a conformational epitope. The two MAbs to the V3 loop region bound to cells infected with HIV-2 ROD. However, as shown above, they recognize overlapping but distinct epitopes. Specifically, the core epitope recognized by 32.2f is two amino acids longer at the N-terminal end than that of 32.7g. We tested both antibodies for binding to envelope glycoproteins by Western blot analysis. Surprisingly, although both MAbs were raised to peptide (circularized) and indeed could be mapped with peptides, MAb 32.2f, unlike 32.7g, failed to bind HIV-2 envelope in Western blots (Fig. 3). This result suggests that there is some local secondary structural requirement for recognition of the epitope by 32.2f.

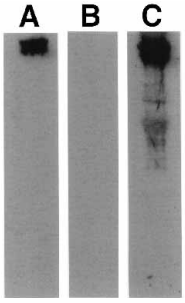


FIG. 3. Western blot analysis of pelleted cell-free HIV-2 ROD virus. Lanes: A, V2 MAb 44.2g; B and C, V3 loop MAbs 32.2f and 32.7g, respectively.

Competition for binding of the MAbs within group A. Group A MAbs (28.3e, 28.8e, and 25.3f) mapped outside V1/V2 and V3 but failed to map to a linear epitope (of 12 amino acids or less in length), and presumably they require some tertiary structure of the HIV-2 ROD envelope in order to bind. To determine if they recognize the same epitope, we tested whether MAbs 28.3e and 25.3f competed with 28.8e for binding to gp105 (ROD) by ELISA. For controls, we tested competition by MAbs from each of the groups as well as a MAb to the V3 domain. The results are shown in Table 2. As expected, none of the MAbs in group B, C, or D competed for binding of 28.8e. Within group A, although unlabelled 28.8e successfully competed with iodinated 28.8e, neither 28.3e nor 25.3f could compete with 28.8e for binding. These results indicate that 28.3e and 25.3f recognize an epitope(s) distinct from 28.8e.

Group A MAbs to conformationally sensitive epitopes do not block binding of sCD4 to virus envelope. MAbs to HIV-1

TABLE 2. Competition studies between MAbs which are dependent on the tertiary structure of the envelope and MAbs to linear epitopes[a]

MAb	Group	% Binding	
		28.8e	44.2g
28.3e	A	102	92
28.8e	A	1	85
25.3f	A	97	147
44.5j	D	96	111
44.5k	B	101	86
25.8c	B	103	111
44.2g	B	83	1
44.1b	B	95	125
44.6i	C	98	131
32.7g	V3 loop	92	100
No competing MAb		100	100

[a] A 10-μg/ml amount of each MAb was mixed with ^{125}I-labelled 28.8e or ^{125}I-labelled 44.2g. Competition for binding to recombinant gp105 was assessed by radioimmunoassaying (see Materials and Methods).

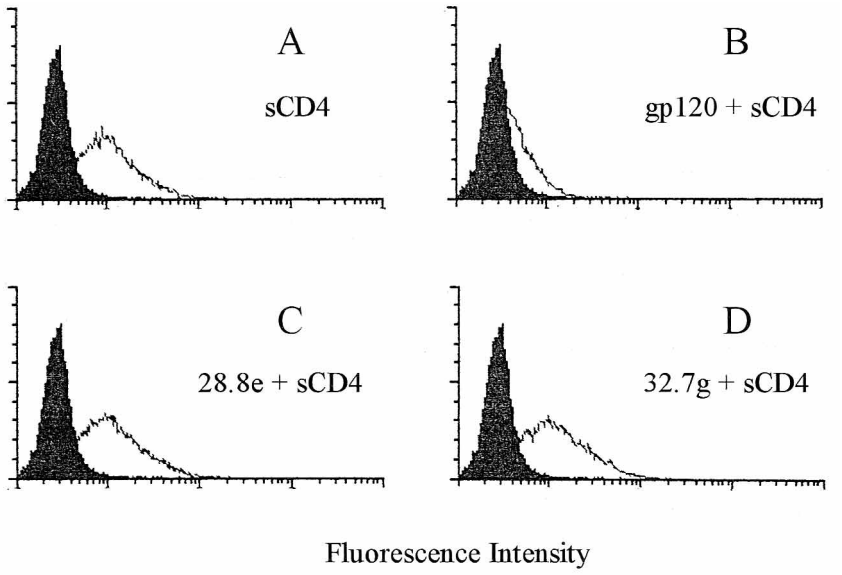


FIG. 4. MAbs to the HIV-2 envelope do not inhibit sCD4 binding. Molt 4 cells chronically infected with HIV-2 ROD expressing high levels of envelope glycoproteins were used to detect sCD4 binding to gp105. (A) HIV-2 ROD-infected molt 4 cells treated with 10 μg of sCD4 alone per ml; (B) recombinant HIV-1 gp120 competing for sCD4 binding. (C and D) failure of 20 μg of 28.8e and 32.7g per ml, respectively, to inhibit sCD4 binding. Bound sCD4 was detected with mouse MAb L120 and then with an anti-mouse IgG FITC conjugate that specifically recognized mouse (but not rat) IgG. Full experimental details are described in Materials and Methods.

envelope glycoprotein that inhibit binding of soluble CD4 (sCD4) to viral envelope or virus have been described elsewhere (15, 27, 28, 64, 67). We designed an assay to test if any of the MAbs described here would block binding of sCD4 to HIV-2 ROD-infected cells. sCD4 was incubated with virus-infected cells in the presence and absence of each MAb. Binding of sCD4 was detected by a mouse antibody specific for domain 4 of CD4 (L120) and then by an anti-mouse fluorescein-conjugated antibody. This conjugate specifically recognized mouse IgG but not rat antibodies. The fluorescence intensity was detected by FACScanning and is presented in Fig. 4. We also checked that sCD4 could be specifically inhibited from binding to infected cells by including envelope glycoprotein (gp120) of HIV-1 that should compete with cell surface ROD envelope for sCD4 binding. Figure 4A shows the binding of sCD4 in the presence of an isotype-matched nonspecific antibody. As expected, gp120 of HIV-1 competed for binding of sCD4 (Fig. 4B). However, none of the MAbs (only 28.8e and 32.7g are shown in Fig. 4C and D, respectively) blocked sCD4 binding to HIV-2 ROD-infected molt 4 cells, even at concentrations of 20 μg/ml. These results indicate that the mechanism of neutralization by these MAbs is not through inhibition of CD4-gp105 binding.

Structural studies of the HIV-2 ROD envelope with the panel of MAbs reveal exposed and hidden domains. The MAbs were used to probe the structure of the HIV-2 ROD envelope. Binding of the MAbs was tested by live- and fixed-cell immunofluorescence. The results are presented in Fig. 5. Assays were done with saturating concentrations of antibody (10 μg/ml). All of the MAbs bound to methanol-acetone-fixed cells. However, MAbs 44.5j, 28.8c, 44.5k, and 44.6i bound weakly or not at all in live-cell immunofluorescence assays or in assays in which cells were fixed in formalin prior to antibody binding, but they bound strongly to methanol-acetone-fixed infected

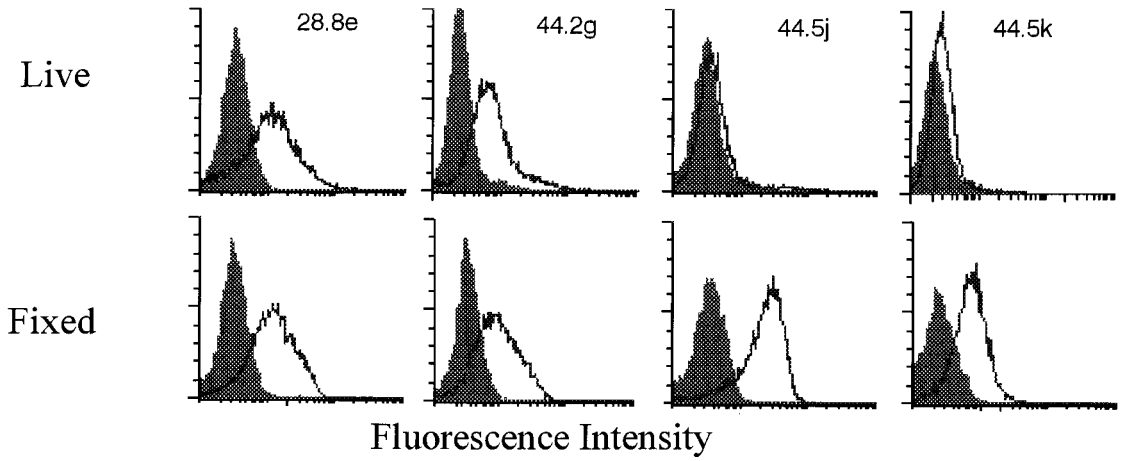


FIG. 5. Serial dilutions of each MAb were tested for binding to live and fixed HIV-2 ROD-infected molt 4 cells. FACS analysis of four MAbs at 10 μg/ml on live and fixed cells. The epitopes for 44.5j and 44.5k are revealed only in fixed cells, whereas epitopes for 28.8e and 44.2g are detected on both live and fixed cells. Exposed amino acids are boxed in Fig. 2.

TABLE 3. MAbs can distinguish conserved and variable domains in the HIV-2 and SIV envelope[a]

MAb (epitope) and virus	Sequence	Result by	
		IF	WB
44.5j (C1)			
ROD	DDYQEITLNVTE	++	+
CBL-22	-----L------	++	+
ISY	-----P------	+	−
32H	G--S-LA-----	−	−
28.8c (V1)			
ROD	SEDTPCARA	++	−
CBL-22	NDTD--IKT	−	−
ISY	NDTD--IQL	−	−
32H	N-TSS-IAQ	−	−
44.5k (V2)			
ROD	GEEETINCQ	+	+
CBL-22	----D---E	−	−
ISY	R--D-D--	−	−
32H	EQ-QM-G-K	−	−
44.2g (V2)			
ROD	FNMTGL	++	+
CBL-22	E-D---	++	+
ISY	------	++	+
32H	------	++	+
			−
44.1b (V2)			
ROD	YSKDVVCET	++	+
CBL-22	--A------	++	+
ISY	--------S	++	+
32H	--T-L---Q	++	+
			−
			+
44.6i (C2)			
ROD	DTNYSGFAPNCS	+	+
CBL-22	-------E----	+	+
ISY	-------E----	+	+
32H	-------M-K--	−	−

[a] The cross-reactivities of the MAbs to HIV-2 isolates CBL-22 and ISY and to SIV MAC strain 32H were determined. Binding to fixed molt 4 cells chronically infected with the appropriate virus strains was determined by immunofluorescence (IF). ++, positive fluorescence (>50% cells positive for fluorescence); +, 10 to 50% positive; −, <10% cells positive. Western blotting (WB) to pelleted virus proteins was tested as described in Materials and Methods. +, positive blotting (see Fig. 3). The results for 44.5j, 28.8c, 44.5k, 44.2g, 44.1b, and 44.6i are shown here with the epitope sequences (from the Los Alamos database [7]). None of the MAbs to conformational epitopes or to the V3 loop could bind any of the isolates tested (apart from the parental strain HIV-2 ROD).

cells. Methanol-acetone fixation permeabilizes the cell membrane, allowing antibody to bind to more immature unfolded forms of envelope protein. Methanol-acetone fixation is also likely to destroy carbohydrate moieties on glycoproteins. These results suggest that the epitopes defined by these domains are at least partially occluded (either by glycosylation or sequestration) on the native gp105. Similarly, 44.2g, 44.1b, and the three MAbs to conformational domains (28.3e, 28.8e, and 25.3f) bound equivalently to fixed or unfixed cells. Likewise, this suggests that these domains are exposed on the native molecule.

It could be argued that the MAbs whose epitopes were determined in this way to be hidden (44.5j, 28.8c, 44.5k, and 44.6i) were of lower affinity and that fixation resulted in antibody gaining access to higher concentrations of antigen. To address this, each MAb was titrated both on live and fixed infected cells. Binding of MAb 28.8e on both live and fixed cells was detected for concentrations of 0.2 μg/ml. Likewise, binding of MAb 44.2g to its linear epitope could be detected for concentrations as low as 1.2 μg/ml on live infected cells and 0.6 μg/ml on fixed infected cells. This result indicates that the increase in concentration of antigen that results from fixation of the virus-infected cells is small. In contrast to the binding of these MAbs, no binding was detected on live infected cells for 44.5j, 28.8c, and 44.5k at concentrations of as high as 10 μg/ml and for 44.6i at concentrations of as high as 5 μg/ml. However, binding of each of these MAbs on fixed infected cells could be detected at 0.6 μg/ml.

TABLE 4. Neutralization titers for rat MAbs to exposed epitopes on HIV-2 SU envelope glycoprotein[a]

MAb	Envelope domain	Neutralization titer (μg/ml)
44.2g	V2	10
44.1b	V2	>20
32.7g	V3	>20
32.2f	V3	2.5
25.3f	Conf.	>20
28.8e	Conf.	0.3
28.3e	Conf.	>20
Isotype matched MAb		>20

[a] The neutralizing activities of MAbs were assessed as described in Materials and Methods; MAbs to occluded epitopes (44.5j, 28.8c, 44.5k, and 44.6i) did not neutralize at antibody concentrations of up to 20 μg/ml. Conf., conformational epitope.

Conservation of MAb epitopes: type and group specificity. We then determined the ability of the MAbs to recognize HIV-2 isolates CBL-22 and ISY and the SIV isolate 32H. Binding was determined by fixed-cell immunofluorescence and by Western blotting (see Materials and Methods). MAbs in group A and both V3 loop MAbs were type specific and bound specifically to the immunizing HIV-2 ROD strain (data not shown). The rest of the results are shown in Table 3, together with available sequence data (Los Alamos database [7]). As might have been expected from published sequences, MAbs 28.8c and 44.5k, which are to occluded V1/V2 epitopes, were type or ROD specific and did not recognize the other isolates tested either by Western blotting or by fixed-cell immunofluorescence. In contrast to this, both of the epitopes of 44.2g and 44.1b to the exposed crown of the loop are group specific and also recognize SIV. The conservation of this region suggests that it may have an important functional role. The epitope for 44.2g is also conserved in HIV-2 strains CBL-21, CBL-23, and ST, as well as in the SIV MAC strain 239 by sequence comparison and by MAb binding (determined by immunofluorescence; data not shown). In contrast, 44.5j (which defines the epitope in C1 [DDYQEITLNVTE]) recognized CBL-22 but failed to recognize ISY or 32H. MAb 44.6i (which maps in a small loop in C2) binds to all of the HIV-2 isolates but not SIV 32H.

MAbs to exposed regions in V2 and V3 and to a conformational epitope are neutralizing. We then tested whether any of the MAbs could neutralize virus infectivity as described in Materials and Methods (Table 4). Three of the MAbs were determined as neutralizing. One of the MAbs, 28.8e, which was shown to recognize a conformational dependent target, was a potent neutralizer and neutralized ROD infectivity at concentrations of as low as 0.3 μg/ml. As expected from binding studies, 28.8e was type specific and did not cross-neutralize other HIV-2 or SIV strains. MAbs to another exposed, conformationally dependent epitope(s) did not neutralize. One of the MAbs to the V2 domain (44.2g) neutralized ROD infectivity at 10 μg/ml. This MAb recognized a conserved epitope and additionally cross-neutralized other HIV-2 strains (ISY and CBL-22) and the SIV SM strain. Interestingly, although

this epitope is conserved in SIV 32H, neutralization of this isolate was not detected, indicating that the epitope may be influenced by amino acids outside this region. In contrast, 44.1b, which also recognized a relatively conserved and exposed epitope on V2, failed to neutralize ROD infectivity. One V3 loop MAb, 32.2f, neutralized virus infectivity, and this antibody was type specific, whereas the other, 32.7g, was nonneutralizing.

DISCUSSION

Many MAbs to HIV-1 envelope gp120 have been exploited extensively to probe its topographical structure. In the absence of a crystal structure, this approach was successful at defining a three-dimensional model of the viral envelope glycoprotein (50). Similar studies with HIV-2 SU envelope have been limited. Here, we describe the production, epitope mapping, and characterization of 11 neutralizing and nonneutralizing MAbs to the SU envelope glycoprotein of HIV-2.

Three MAbs, 28.8e, 28.3e, and 25.3f, were defined by competition studies to bind to at least two nonoverlapping epitopes. These MAbs were strain specific yet mapped to regions outside the V1, V2, or V3 domain. In addition, they failed to bind to any of 80, 12-mer overlapping peptides representing the entire HIV-2 ROD envelope. Although it is still possible that the epitopes recognized by these MAbs need a linear peptide longer than 12 amino acids to bind, it is likely that they require some tertiary structure of the envelope glycoprotein. The lack of binding in Western blot analysis of severely denatured proteins supports this conclusion. One of these MAbs, 28.8e, efficiently neutralized ROD infectivity for $CD4^+$ T cells. Other neutralizing MAbs to conformational domains include those to the CD4-binding site on gp120 of HIV-1 (64, 70). However, it is unlikely that 28.8e recognizes the CD4-binding site of HIV-2, since it failed to block the binding of sCD4 to viral envelope glycoprotein expressed on virus-infected cells. Most but not all neutralizing MAbs to discontinuous epitopes on the HIV-1 CD4-binding domain cross-neutralize other strains (64, 70). However, MAb 28.8e is type or ROD specific. Neutralizing MAbs to conformationally dependent epitopes in the V2 domain of HIV-1 (22, 26, 47, 51) and to an epitope on SIV that includes the V3 loop but not the CD4-binding site have also been described elsewhere (32, 37). However, the mapping studies described here demonstrate that neither the V2 nor the V3 region is involved in the 28.8e epitope. A neutralization epitope in the V4 region of the SIV envelope has been described elsewhere (72), and we cannot exclude V4 from the epitopes of 28.8e, 28.3e, and 25.3f.

Six other MAbs raised to recombinant envelope glycoprotein mapped to novel linear epitopes in C1, V1, V2, C2, and V3 (Fig. 2; Table 1). The epitope for MAb 44.5j, which mapped within C1, is conserved among the HIV-2 isolates tested. However, the central residue within this epitope, isoleucine, is variable. A conservative substitution of this residue to leucine in CBL-22 still allowed recognition by 44.5j. Substitution of this residue for a proline, as in ISY, resulted in reduced binding. Together, these data suggest that this residue is not directly involved in the binding site, although nonconservative substitutions may result in distortion of the epitope. In contrast, the 44.5j epitope has four substitutions in SIV 32H, compared with ROD, and this results in loss of binding. Analysis of the exposure of this epitope in the native envelope indicated that it is at least partially occluded, in agreement with the data suggesting that the equivalent region on HIV-1 gp120 glycoprotein is hidden (50).

44.2g, one of the four MAbs to linear epitopes in the V1/V2 domain, was neutralizing. Linear epitopes in the V2 region have been previously shown to be targets for neutralization of both HIV-1 and SIV (3, 22, 26, 38, 47, 51). The MAb 44.2g described here recognizes an epitope defined by amino acids FMNTGL, which is equivalent to neutralization targets for HIV-1 (65, 74) and SIV (3, 38). Although weak, 44.2g could neutralize both HIV-2 strains CBL-22 and ISY and one SIV strain from sooty mangabey, SIV SM. Interestingly, although 44.2g bound to SIV 32H, this virus was not neutralized, suggesting that regions outside the epitope influence neutralization. Regions outside the critical epitope might affect the exposure and the availability of this region on virions. The base of the V2 domain is buried on the native molecule but is extremely variable. Particular amino acid substitutions in this region may result in local distortions, altering the conformation of the 44.2g epitope. Furthermore, the tip of the V2 domain is relatively conserved compared with the base. It is possible that amino acid changes at the base of the loop influencing V2 conformation result in neutralization escape.

Two other MAbs to linear, nonoverlapping V2 epitopes were also generated. The epitope defined by MAb 44.5k at the base of the V2 loop and buried is highly variable, and this MAb binds only to HIV-2 ROD. Likewise, the 25.8c epitope on V1 is variable and hidden. In contrast, the exposed epitopes defined by 44.1b as well as 44.2g at the V2 tip are more conserved. These results also indicate that the overall topology of this region is similar to that of HIV-1 (50) and that the conservation of the V2 tip may indicate it to be functionally important.

Two MAbs were raised to a V3 loop peptide (circularized by disulfide-bonded cysteines). One of these MAbs, 32.7g, failed to neutralize infectivity. The second MAb, 32.2f, was a potent neutralizer of infectivity for C8166 cells. Both MAbs map to the same V3 loop region. MAb 32.2f bound a linear peptide, LMSGHVFHSHYQ, whereas 32.7g recognized a shorter sequence, SGHVFSHSHYQ, contained within the 32.2f determinant. Both MAbs to the V3 loop are strain specific and bind only to the immunizing strain HIV-2 ROD. Interestingly, 32.7g but not 32.2f could detect envelope glycoprotein in Western blot analysis. This result suggests that the 32.2f epitope depends on some secondary structure that can be formed in a peptide preparation but lost during preparation for Western blot analysis. The amino acid sequence within this epitope would be expected to form an alpha-helical secondary-structure motif which may be required for the binding site of MAb 32.2f. The V3 loop of HIV-1 is long established as a neutralization determinant of HIV-1 on T-cell-line-passaged viruses (23, 30, 31, 43, 45, 57) where the V3 loop is exposed (50). On primary, low-passage HIV-1 strains, the V3 loop may be more cryptic and protect against antibody neutralization (6, 49, 68). One human MAb described by Moore et al. (52) that recognized residues on either side of but very close to the V3 loop tip was broadly neutralizing for primary HIV-1 strains, suggesting exposure of at least the V3 loop tip. Peptides representing the V3 loop of SIV did not induce neutralizing antibodies (33, 38), although more recent studies show that the V3 loop of SIV is involved in the conformation of the epitope recognized by a MAb that neutralizes SIV (32). The role of the V3 loop of HIV-2 as a neutralization target has previously been controversial; however, Matsushita et al. (44) recently reported a weakly neutralizing MAb directed to the HIV-2 ROD V3 loop. The core epitope of this MAb is HYQ, which overlaps both of the epitopes described here as well as that described by Björling et al. (5).

Finally, MAb 44.6i binds to an epitope within a highly conserved double-loop structure of unknown function (Fig. 2) that is conserved in HIV and SIV isolates (29) (Table 3). It is predicted to be highly glycosylated (29) and contains three potential glycosylation sites, two of which are within the antibody-binding site. Indeed, in our immunofluorescence analysis, we could detect little or no binding of this MAb to live infected

cells. Binding occurred only after fixing the cells in methanol-acetone or by denaturing during Western blot analysis. While we cannot exclude that this epitope is buried in the native molecule, it seems likely that it is masked by sugar residues.

In summary, we have generated a panel of 11 MAbs to the SU glycoprotein of HIV-2 ROD. Three of the MAbs recognized at least two conformation-dependent epitopes; however, unlike similar MAbs to HIV-1 envelope glycoprotein, they do not block the binding of sCD4 to the virus envelope. Other MAbs generated to the viral envelope were mapped to linear epitopes in C1 (44.5j) and V1 (28.8c), as well as three within the V2 domain (44.5k, 44.4g, and 44.1b). MAb 44.6i binds to a highly conserved double-loop structure in C2 with three potential glycosylation sites. Finally, we have generated two MAbs to a peptide representing the V3 domain. Both V3 MAbs recognize distinct but overlapping linear epitopes. MAbs that neutralized recognized exposed epitopes on V2 and V3 and a domain dependent on conformation.

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